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Identifying molecular markers for the early detection of pancreatic neoplasia

Michael Goggins, MD

Departments of Pathology Medicine, and Oncology The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins Medical Institutions, Baltimore, MD 21205-2196

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The benefits of detecting early pancreatic neoplasia

Pancreatic ductal adenocarcinoma is the fourth leading cause of cancer death in the USA and has the poorest survival rate of the common cancers (~2%)¹. The poor survival is attributed to the advanced stage of disease most patients (~85%) reach at diagnosis². Even among patients that have resectable pancreatic cancers, currently only ~15% will have the earliest-stage cancers (T1, or T2 tumors without lymph node metastases), and pancreatic cancer survival is better in patients with the earliest-stage tumors^{3,4}. Thus, only 2-3% of all patients diagnosed with pancreatic cancer present at the earliest-stage. Patients with pancreatic cancer that do undergo surgical resection achieve a 5-year survival of 15-40%⁵. The late diagnosis of pancreatic cancer is usually because patients present with advanced disease. Sometimes patients present with non-specific symptoms, resulting in delays to referral to specialized diagnostic services^{2,6}. In patients in whom pancreatic cancer maybe suspected, imaging and marker studies may not be able to distinguish between pancreatic adenocarcinoma and non-neoplastic pancreatic diseases such as chronic pancreatitis and non-neoplastic pancreatic cysts. For these reasons, much effort has gone into identifying accurate markers of pancreatic cancer to facilitate the early diagnosis of pancreatic cancer.

The standard serum marker, sialylated Lewis^a blood group antigen CA19-9, is widely used but its use is limited to monitoring responses to therapy, not as a diagnostic marker⁷⁻¹¹. CA19-9 is not used for diagnosis because false-positive results are common among patients with benign pancreaticobiliary disorders. CA19-9 also has imperfect sensitivity. Some 5-10% of the populations do not express Lewis antigens and do not have detectable CA19-9 levels⁷⁻⁹ and CA19-9 is elevated in only ~65% of individuals with a resectable pancreatic cancer¹¹. CA19-9 is also unable to reliably differentiate patients with pancreatic cancer from those with chronic pancreatitis; up to 40% of patients with chronic pancreatitis will have an elevated CA19-9¹². Improved serum markers of pancreatic cancer could facilitate the early detection of pancreatic cancer. Perhaps the best illustration of the benefit of diagnosing pancreatic neoplasia early comes from recent studies in which high-risk populations have been screened using

Corresponding author: Michael Goggins, M.D., The Sol Goldman Pancreatic Cancer Research Center, The Johns Hopkins Medical Institutions, Department of Pathology, 1550 Orleans St, Baltimore, MD 21231, Telephone: (410) 955-3511, Fax: (410) 614-0671 Email: mgoggins@jhmi.edu.

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endoscopic ultrasound to detect early pancreatic neoplasia. However, screening for pancreatic cancer with serum CA19-9 in both asymptomatic populations and populations at high risk for pancreatic cancer results in high false-positive rates ^{13, 14}.

Targeting high risk groups for the early detection of pancreatic neoplasia

Recent studies demonstrate that many individuals at high-risk of developing pancreatic cancer can be identified based on their family history ¹⁵⁻¹⁹. However, the identification of individuals most at risk of developing pancreatic cancer will be improved once the genetic alterations that predispose to inherited forms of pancreatic cancer are identified ¹⁹⁻²⁷.

While it is important to target high-risk individuals with a family history of pancreatic cancer, such a strategy does not facilitate the early detection of pancreatic cancer in individuals with sporadic forms of pancreatic cancer. One strategy that has been considered as a way of identifying early pancreatic cancer in these groups is to determine if the onset of diabetes mellitus in patients with otherwise clinically silent pancreatic cancer would herald the disease while it is still curable. Interest in this approach comes with the recognition that most patients with pancreatic cancer have either impaired glucose tolerance or diabetes mellitus at the time of diagnosis ²⁸. It is not yet known if rapid evaluation of the pancreas in someone presenting with diabetes and an otherwise unsuspected pancreatic cancer would improve their chances of survival, but this question could be answered with prospective studies.

Recent screening studies of individuals at high-risk of developing pancreatic cancer based on their family history ¹⁵⁻¹⁹ have demonstrated that many such individuals have asymptomatic pre-invasive pancreatic neoplasms visible by careful endoscopic ultrasound (EUS) and CT scanning of the pancreas ²⁹⁻³¹. This is the optimal goal of screening; to identify precancerous neoplasia so affected individuals can be treated before they develop an invasive cancer. The surgical options for those with pancreatic neoplasms has been to resect the neoplasm as one would for patients with sporadic disease (pancreaticoduodenectomy or tail pancreatectomy) ²⁹, or to perform a total pancreatectomy, as patients are at risk of developing new neoplasms ³⁰. Most of the pancreatic masses treated by surgical resection have been intraductal papillary mucinous neoplasms (IPMNs), an increasingly recognized precursor to pancreatic ductal adenocarcinoma ³². Cure rates are very high after resection of IPMNs that do not have an associated infiltrating ductal pancreatic adenocarcinoma ^{33, 34}. These results suggest that screening high-risk individuals to detect pancreatic neoplasia will be an important part of the management of these individuals, although further study is needed to determine the optimal screening strategy and to determine which high-risk populations should be targeted for screening. Ultimately, if early detection efforts are able to detect and treat precancerous lesions of the pancreas, the incidence of pancreatic cancer could be reduced.

However, one of the limitations of pancreas imaging is that often pancreatic lesions are detected that are of uncertain significance. Determining the nature of imaging abnormalities can be difficult as fine needle aspiration cytology is often inconclusive. Yet patients can develop invasive pancreatic cancers that are only 1 cm in size, although most pancreatic lesions of this size are either benign neoplasms of low malignant potential or simple cysts. Molecular markers are needed to help determine the nature of pancreatic lesions identified by imaging during the screening of high-risk individuals. As well as pancreatic ductal adenocarcinoma and IPMNs, high-risk individuals also develop microscopic PanIN (pancreatic intraepithelial neoplasia). PanINs are small neoplasms 5mm or less more often found in the head of the gland that are thought to be the commonest precursor to invasive pancreatic ductal adenocarcinoma ³². Unfortunately, most precursor lesions, particularly PanINs are only reliably identified after their surgical resection. Interestingly, the technique of tru-cut biopsies of the pancreas have been performed to better evaluate pancreas pathology ³⁵. While the safety of the technique is

still undergoing evaluation, tru-cut biopsies can aid in the differential diagnosis of pancreatic lesions and may be particularly helpful for evaluating PanIN. Since many healthy individuals have low-grade PanIN that will never progress to a clinically important neoplasm³⁶, markers are needed not only to help differentiate neoplastic from non-neoplastic pancreatic lesions, but also to indicate the presence of microscopic high-grade PanIN that might herald future pancreatic cancer risk. Molecular markers that could identify PanIN or IPMN non-invasively have not yet been identified, though many of the molecular changes found at lower prevalence in pancreatic ductal adenocarcinomas are found in PanINs and IPMNs. Therefore, some markers that are being investigated for pancreatic cancer diagnosis could be used to help predict the presence of PanIN or IPMNs in affected pancreata.

Because of their size and their non-invasive nature, microscopic precancerous lesions (PanINs and IPMNs) are unlikely to be detected using serum markers. Pancreatic juice analysis is being investigated as a source of markers of pancreatic neoplasia, analogous to other “local” diagnostic marker strategies such as sputum analysis for lung neoplasia. Pancreatic juice can be collected during upper gastrointestinal endoscopy after secretin infusion, and can be used to analyze markers in patients that have microscopic neoplastic foci. In contrast, fine needle aspirates (FNAs) are best used to sample focal lesions visible by imaging. Since cytological interpretation of FNAs obtained from pancreatic masses is difficult, molecular markers are also being investigated as a means to improve the diagnostic yield of FNAs^{37, 38}. Because of its higher concentrations of DNA and other molecules released from pancreatic cancers, molecular alterations of pancreatic neoplasia are more readily detected in pancreatic juice than elsewhere. For example, mutated *K-ras*, mutated *p53*³⁹⁻⁴², and telomerase⁴³, are readily detected in pancreatic juice compared to other sites such as plasma, duodenal fluid or stool.

Recent developments in pancreatic cancer markers

A large number of protein- and DNA-based markers are under investigation as pancreatic cancer markers. Most of these markers are still in phase I or II studies, that is their diagnostic potential is still undergoing evaluation and validation⁴⁴. Some markers that show promise in distinguishing samples from patients with pancreatic cancer from healthy controls but fail to distinguish samples arising in the setting of chronic pancreatitis, thereby limiting their diagnostic potential.

Serum Markers

Many markers of pancreatic cancer that have been developed in the past have targeted carbohydrate antigens of MUC-1 in an attempt to improve on the performance of CA19-9 which targets the sialylated Lewis^a antigen on MUC-1⁴⁵. While none of the previous assays used to detect MUC-1 carbohydrate epitopes proved to be any superior to CA19-9, investigators continue to evaluate this target using different antibodies to carbohydrate or other epitopes of MUC-1. In this regard, a described antibody, PAM4, detects pancreatic cancer MUC-1 proteins more specifically than MUC-1 expressed in other cancers, and an ELISA to detect circulating MUC-1 epitopes is more sensitive and specific than CA19-9 in initial studies for identifying patients with pancreatic cancer⁴⁶.

High-throughput discovery microarray platforms for gene expression profiling⁴⁷⁻⁶⁰ have led to the identification of several new marker candidates for pancreatic cancer. For example, one protein expressed in most pancreatic cancer tissues and not in normal pancreas is mesothelin⁶¹. An ELISA has been developed to detect circulating mesothelin demonstrating elevated levels in patients with ovarian cancer over controls⁶². A soluble variant of mesothelin is overexpressed in ovarian and other cancers, and can be detected in serum⁶³, but this form of mesothelin is not generally found in pancreatic cancer sera (Hellstrom and Goggins, unpublished observations). In addition, mesothelin expression in primary pancreatic cancers

can be detected in fine needle aspirates of suspicious pancreatic lesions suggesting that mesothelin and the protein products of several other overexpressed genes may be useful as aids to cytopathological diagnosis³⁷. Mesothelin has also been shown to act as a tumor antigen⁶⁴. Several secreted proteins including MIC-1^{65, 66} and osteopontin⁶⁷ are overexpressed in primary pancreatic cancers and are elevated in the serum of patients with pancreatic cancers.

A recent study found that serum macrophage inhibitory cytokine 1 (MIC-1) is a more sensitive marker of pancreatic cancer than CA19-9⁶⁶. Ninety percent of the patients with resectable pancreatic cancer had MIC-1 levels greater than 2 standard deviations above age-matched controls whereas only 62% had elevated CA19-9. And unlike CA19-9, MIC-1 elevations were independent of TNM stage: 6 of 7 patients with T1 or T2 cancers had elevated MIC-1 whereas only 2 of 7 had elevated CA19-9⁶⁸. MIC-1 was no better than CA19-9 in distinguishing patients with chronic pancreatitis from those with pancreatic cancer. These results suggest that serum MIC-1 could be particularly helpful in the early detection of pancreatic cancer in high-risk populations as part of their pancreatic screening protocols^{29, 30}. MIC-1 is a member of the TGF- β superfamily first identified in the setting of macrophage activation⁶⁹. It is also known as placental TGF- β ⁷⁰, prostate-derived factor⁷¹, growth/differentiation factor 15/MIC-1⁷², and placental bone morphogenetic protein⁷³. MIC-1 is overexpressed in several cancer types including pancreas, colon, prostate, breast and gastric cancers⁷⁴⁻⁷⁶ and thus may have utility in the diagnosis of other cancers⁷⁷.

Proteomics

A variety of proteomics approaches have also been utilized in an attempt to identify protein markers of pancreatic cancer⁷⁸⁻⁸¹. Several groups have identified protein fragments in serum by SELDI (surface enhanced laser desorption ionization) and some of these protein fragments appear to perform as well or better than serum CA19-9 as a diagnostic marker^{82, 83}. Using SELDI as a discovery platform has the advantage that many patient samples can be profiled in single experiments, but it has also become evident that there can be problems with reproducibility using SELDI⁸⁴⁻⁸⁷ unless appropriate technical and analytical measures are taken^{86, 87}. More recent studies have utilized multi-center designs and have validated novel proteins by demonstrating that the same proteins can be detected in the same samples sets in different laboratories^{88, 89}. Another mass spectrometry approach utilizes MALDI (matrix associated laser desorption ionization) and has been used to identify pancreatic cancer proteins in serum.⁹⁰ One interesting approach utilizes stable isotope labeling with amino acids in cell culture (SILAC) to compare the proteins secreted pancreatic cancer cells from non-neoplastic pancreatic ductal cells⁸⁰. Pancreatic juice has also been investigated as a source of pancreatic cancer markers. Investigators have used 1-dimensional gel electrophoresis followed by liquid chromatography tandem mass spectrometry to identify potential markers of pancreatic cancer⁹¹, others have used SELDI⁹². Several groups have applied proteomics approaches to pancreatic cancer cells rather than secondary sources such as serum and compared them with normal and inflamed pancreas^{93, 94}. This approach has the advantage of evaluating proteins from the source tissue but since primary tissues reflect a mixture of multiple cell types, determining the relative abundance of proteins in one tissue versus another can be a challenge. On the other hand, many of the proteins identified from serum proteomics studies are high-abundance proteins, and many of the differentially expressed proteins identified are not suitable for diagnostic use. Thus, despite much effort by proteomics investigators to identify markers of pancreatic and other cancers by profiling proteins, only limited success has been achieved to date in identifying markers that will ultimately be useful as diagnostic tests. This limited success highlights the challenge of interrogating the large numbers of proteins in the proteome (hundreds of thousands of proteins and protein modifications), their complex biology, the vexing chemical properties of proteins that can make them hard to separate, and the influence of physiological states, sample collection, and sample handling can have on protein profiles

95, 96. Another problem that may arise when undertaking marker discovery studies is bias, wherein markers are identified that purport to reflect differences between patients with disease vs. those without, but merely reflect trivial differences in the study populations such as patient age, or differences that arise from differences in sample handling or data interpretation⁹⁷. Despite these problems, recent proteomics studies have led to a greater understanding of the “peptidome”, the small peptides of many proteins found in the circulation and in other compartments that result from protease digestion that arise in inflammatory and neoplastic states⁹⁸.

Markers for pancreatic juice analysis

Genetic and epigenetic markers have been extensively investigated in pancreatic juice. Protein markers require accurate quantification, and their levels may be quite variable in pancreatic juice in normal individuals, whereas initial studies of genetic and epigenetic markers anticipated that the mere detection of such alterations would have some diagnostic value. Some of the genetic and epigenetic markers that have been investigated in pancreatic juice include mutant *K-ras*, *p53* mutations, DNA methylation alterations, and mitochondrial DNA mutations.

Genetics markers

Mutant *K-ras* has been extensively investigated as a marker of pancreatic adenocarcinoma because mutations are present in ~90% of pancreatic ductal adenocarcinomas, and can be readily detected using molecular assays as they are generally limited to one codon. *K-ras* mutations are present not only in invasive pancreatic cancer, they occur in patients with chronic pancreatitis, in individuals who smoke, and in PanINs from patients without pancreatic cancer^{42, 99-102}. Mutant *K-ras* can also be detected in the blood of patients with pancreatic cancer more often in patients with advanced stage cancers^{39, 40}. While these studies have limited the enthusiasm for mutant *K-ras* as a diagnostic marker, improved assay methods could facilitate that the quantification of mutant *K-ras*. Mutation-containing DNA is very difficult to detect in the presence of abundant wild-type DNA. Recently, however, several sensitive assays have been developed that can accurately quantify levels of mutant DNA. One method, termed LigAmp (because it involves DNA ligation and PCR amplification) has been used to quantify *K-ras* in pancreatic juice¹⁰³. Another molecular strategy that can be used to detect single base pair mutations such as occur in *K-ras* is BEAMing (on the basis of four of its principal components: **B**eads, **E**mulsion, **A**mplification, and **M**agnetics)^{104, 105}. The BEAMing technology converts single DNA molecules to single magnetic beads, each containing thousands of copies of the sequence of the original DNA molecule. Mutant molecule specificity is determined using fluorescent labeled oligonucleotides and the number of mutant DNA molecules in the population is determined by quantifying fluorescence using flow cytometry. BEAMing has been used to demonstrate that patients with colorectal cancers frequently have circulating mutant *APC* fragments. Thus, with newer quantitative assays, it remains possible that quantifying mutant *K-ras* levels could improve the diagnostic utility of mutant *K-ras*.

TP53 gene mutations generally occur relatively late in the neoplastic process towards invasive pancreatic cancer, and the detection of *TP53* gene mutations has been widely investigated as a potentially specific diagnostic marker in various cancers. In pancreatic ductal adenocarcinoma, *TP53* gene mutations are found in ~70% of invasive cancers⁴². Although a few nucleotide hot spots of *TP53* gene mutation are known to exist, mutations occur throughout the gene¹⁰⁶. Most studies of *TP53* mutations as a cancer marker have used assays such as chip technologies, single-strand conformational polymorphism, and temperature gradient capillary electrophoresis that have the potential to identify the complete spectrum of *TP53* gene mutations. In one study, investigators reported the presence of *TP53* gene mutations, in pancreatic juice samples and in brush cytology specimens of 40%-50% of patients with

pancreatic cancers¹⁰⁷. Recently, investigators have used a combined marker strategy in an attempt to improve the molecular diagnosis of pancreatic cancer¹⁰⁸. Their marker panel included mutant *K-ras*, methylated *p16* and a functional yeast assay for detecting *p53* mutations, and of these markers the presence of *p53* mutations was the most specific. Thus, the detection of P53 mutations in pancreatic juice has the potential to be a useful diagnostic strategy if improvements in mutation detection technology can enable facile and accurate detection of such mutations at low concentration.

Recently, several cancer-causing mutations have been identified in several cancers types using high-throughput DNA sequencing strategies analyzing suspected mutation targets. For example, kinome sequencing has involved sequencing the coding regions of several hundred kinase genes, such as the tyrosine kinases. This approach has led to the discovery of mutations in several kinase genes including tyrosine kinase, tyrosine phosphatase and lipid kinase genes in colorectal cancer¹⁰⁹⁻¹¹². Similarly, sequencing known components of an important signaling pathway led to the identification of *BRAF* mutations in melanomas¹¹³. While the genes identified as mutated using this approach have not yet been shown to be mutated in pancreatic cancers, the high-throughput sequencing approach directed at these other important classes of candidate genes is likely to identify additional genes mutated in pancreatic cancers that could serve as targets for early detection approaches.

Epigenetic markers

Pancreatic cancer is an epigenetic disease as well as a genetic disease¹¹⁴⁻¹¹⁷. Numerous genes undergo aberrant methylation during pancreatic cancer development and methylation of many of these genes is rarely detected in non-neoplastic pancreatic tissues. These genes include *p16*^{118, 119}, *RELN*¹²⁰, *DAB1*¹²⁰, *ppENK*^{121, 122}, *Cyclin D2*¹²³, *SOCS1*¹²⁴, *SPARC*¹²⁵, *TSLC1*¹²⁶, and others^{122, 127}. Many of these genes are aberrantly methylated in a high proportion of pancreatic cancers and can be detected with methylation specific polymerase chain reaction (PCR) making them potentially attractive for early detection. Indeed, initial studies indicate that the detection of aberrantly methylated genes in the pancreatic juice of patients with pancreatic cancer is a promising diagnostic strategy^{121, 128-130}. Further discovery of aberrantly methylated genes may help identify genes that have greater specificity for cancer, in other words, genes that are not methylated in non-neoplastic diseases. In this regard, newer methods for detecting aberrant methylation have been developed that utilize microarrays containing promoter sequences. These microarrays can be used to identify methylated promoters using methylation-sensitive restriction enzymes or antibodies to methylated cytosine nucleotides^{131, 132}.

The emergence of chip technologies has also facilitated investigations into the diagnostic utility of mitochondrial mutations. Mitochondrial mutations are commonly found in multiple cancers types¹³³⁻¹³⁷. One advantage of mitochondrial DNA as a marker for cancer is that each cell has many more copies of the mitochondrial genome than of nuclear DNA, and the amount of mitochondrial DNA in cancer cells is several times more abundant than it is in normal tissues^{133, 134, 136, 138}. A "MitoChip" has been developed to interrogate the mitochondrial genome,¹³⁵ and initial studies suggest that this chip can be used to detect mitochondrial mutations in pancreatic juice samples obtained from patients with pancreatic cancer¹³⁵.

microRNA alterations in pancreatic cancer

MicroRNAs are recently described small RNAs that regulate gene expression. MicroRNAs are formed from larger RNA transcripts that are degraded by an enzyme known as DICER, then associate with an RNA-induced silencing complex (RISC) and bind to the 3'untranslated regions of many genes thereby inducing RNA degradation or translational repression. Over 300 hundred microRNAs have been identified and widespread alterations in these microRNAs

have been identified in various types of cancer¹³⁹⁻¹⁴³. While the expression of most microRNAs appear to be reduced in cancer, several are overexpressed and could be potential targets for early detection assays.

In summary, many new protein-, DNA-, and RNA-based markers are under investigation as markers of invasive pancreatic cancer. While some of the newly described markers are promising, many require further validation using optimal laboratory methods and suitable patient populations before they can be considered ready for use in clinical settings. Since the best chance of cure from pancreatic cancer is when the cancer is at an early stage, clinical studies involving pancreatic cancer markers must focus their efforts on patients with early stage pancreatic cancer. The successful results of recent screening studies demonstrating that pre-invasive neoplasms can be detected and cured using accurate pancreatic imaging tests has highlighted the importance of ongoing studies to identify individuals at increased risk of developing pancreatic neoplasia. These studies also highlight the need to also develop accurate markers of pre-invasive pancreatic neoplasms that could help predict future cancer risk among individuals undergoing screening.

Abbreviations

IPMN, Intraductal papillary mucinous neoplasm; ERCP, endoscopic retrograde cholangiopancreatography; EUS, endoscopic ultrasound; MIC-1, macrophage inhibitory cytokine-1; PanIN, pancreatic intraepithelial neoplasia.

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